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# Structural Biology in Antiviral Drug Discovery

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## Abstract

Structural biology has emerged during the last thirty years as a powerful tool for rational drug discovery. Crystal structures of biological targets alone and in complex with ligands and inhibitors provide essential insights into the mechanisms of actions of enzymes, their conformational changes upon ligand binding, the architectures and interactions of binding pockets. Structure-based methods such as crystallographic fragment screening represent nowadays invaluable instruments for the identification of new biologically active compounds. In this context, three-dimensional protein structures have played essential roles for the understanding of the activity and for the design of novel antiviral agents against several different viruses. In this review, the evolution in the resolution of viral structures is analysed, along with the role of crystal structures in the discovery and optimisation of new antivirals.

## Keywords

X-ray crystallography, PDB, structure-based drug discovery, antiviral agents.

## Abbreviations

DENV	Dengue virus
EV71	Enterovirus 71
FOAMV	Simian foamy virus
HCV	Hepatitis C virus
HHV	Human herpesvirus
HIV-1	Human immunodeficiency virus 1
MLV	Murine leukemia virus
NV	Norovirus
RSV-SRA	Rous sarcoma virus
SARS-CoV	Severe acute respiratory syndrome-related coronavirus, Human SARS coronavirus

## 1. Introduction

Knowledge of the three-dimensional structures of proteins has been long recognised as a powerful tool to accelerate drug discovery, providing information about target shape, hydrophobic and hydrophilic behaviours of macromolecules and interactions with substrates [1]. Since 1934, when the first X-ray diffraction structure was reported for pepsin, this method emerged as an invaluable source of detailed

and reliable information about protein structure, representing a significant step forward in comparison with previously used physical or chemical methods [2].

The idea that three-dimensional structural information could be useful in defining topographies of the complementary surfaces of ligands and their protein targets raised in the early 1980s, when scientists started to use this information to optimise potency and selectivity of lead compounds [3]. Structural details gathered from a target structure in the presence of unique ligands can provide fundamental insights on the geometric fit of these compounds into the binding site, on the binding of active conformations, on molecular electrostatic potentials and on hydrophobic interactions [4]. From 1980 to nowadays, the applicability of structural biology was extended to the assessment of target druggability, to the identification of hits by virtual screening with structure-based virtual screening methods, to target identification by structure-sequence homology recognition [5].

During the last three decades, the application of structural biology to drug discovery followed a classical path. Structure-based became extremely fashionable during the 1980s, as a consequence of the publication of the structures of the first important drug targets. Later on, due the limited number of protein structures available and to the cost and time required to set up the crystallization process, the use of these rational approaches dropped in favour of high-throughput screening methods and combinatorial chemistry [5]. The successful story of HIV protease inhibitors [6,7] and influenza antiviral drug Relenza [8] led to a renewed interest in target structure-driven drug discovery. Furthermore, the numerous advances in science and technology promoted a faster and less expensive application of structural biology, increasing the speed of macromolecular structure determination, increasing the resolution of new crystal structures and allowing smaller amounts of protein and fewer crystals to be required to solve a structure [9].

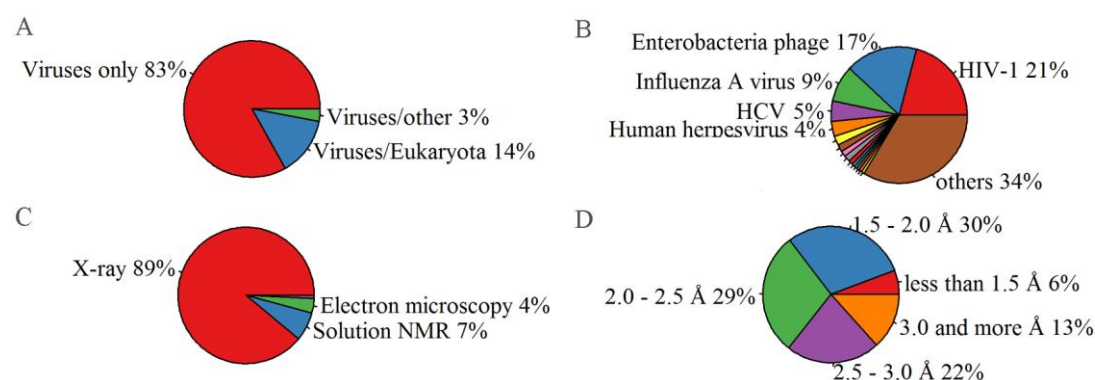
One of the most recent applications of structural biology is crystallographic fragment screening [1]. The low affinity for the target that characterises chemical fragments has made them unsuitable for classical high throughput screening, but the advances in high throughput NMR and crystallography have allowed the use of structural information of protein–fragment complexes, which provide reliable proof of binding pockets and hit binding mode, and give clear indications on how the fragment structures can be optimised into potent lead compounds [10]. These characteristics make fragments very attractive starting points for iterative medicinal chemistry optimization [1].

Several biologically active compounds discovered by structure-based design are now drugs in the market, confirming the crucial role played by structural biology in drug development [11], while protein structure determination has become one of the earliest and most crucial steps for many drug-discovery programs of pharmaceutical companies [1].

## **2. Viral structures in the Protein Data Bank**

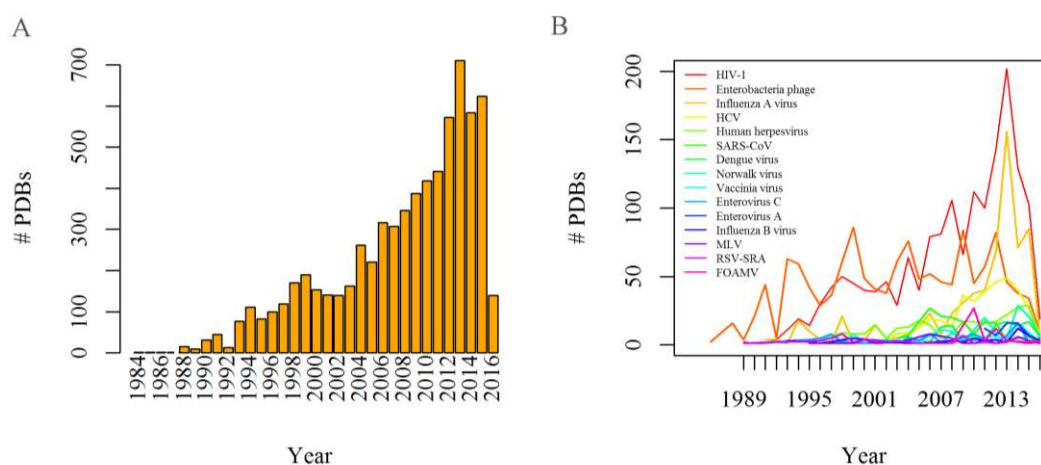
The Protein Data Bank (PDB) was established in 1971 as a curated archive that evolves with new developments in structural biology [12]. The holdings in the PDB continue to grow and the usage of PDB data is also growing. In 2015, 526 million downloads (ftp and website) of data occurred from the PDB sites, compared to 226 million downloads in 2008. Download statistics for the overall archive and for individual entries are available from the wwPDB website (<http://www.wwpdb.org/stats/download> ).

The first atomic viral structure was published less than 40 years ago [13], while now about 7,852 viral related structures are available in the PDB from about 475 different virus, more than 80% of them containing viruses only (**Figure 1A-B**). Most viral structures in the PDB (around 89%), have been determined using X-ray crystallography (**Figure 1B**). Since the establishment of this archive, the number of structure depositions has grown steadily. The average resolution of X-ray structures has remained constant at about 2.54 Å (**Figure 1C**). However, with the large volume of data available, there are now substantial numbers of structures determined to a high resolution (below 1.5 Å), including at least one viral structure [14]. At the same time, as more large macromolecular machines are being studied using X-ray methods, there are many examples of low resolution structures (above 3.5 Å) [15-17].



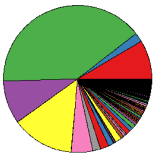
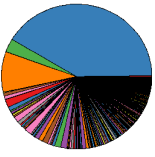
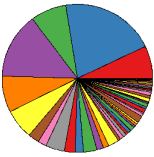
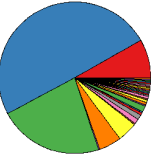
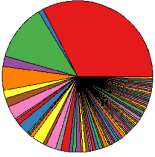
**Figure 1.** Statistical details of 7,852 PDB entries considered. RCSB Protein Data Bank was used to retrieve the viral PDB entries using “TAXONOMY is Viruses” as a search string. (A) Taxonomy, (B) detailed taxonomy, (C) experimental method, and (D) resolutions.

Over the years the number of viral PDB entries has increased at an increasingly faster rate (Figure 2A). Analysis of the taxonomy in the PDB shows that the most studied viruses are, respectively, HIV-1, Enterobacteria phage, Influenza A virus, HCV, Human herpesvirus, SARS-CoV, Dengue virus, Norwalk virus, Vaccinia virus, Enterovirus C, Enterovirus A, Influenza B virus, MLV, RSV-SRA and FOAMV (Figure 2B). This is most likely due to the important roles that these viruses play in biomedical research.

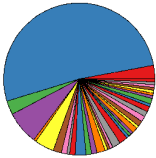
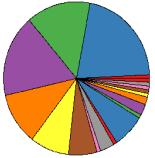
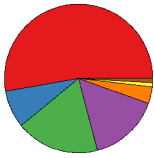
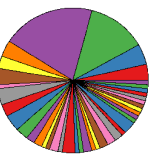
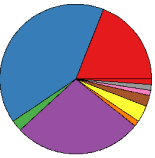


**Figure 2.** Growth in the numbers of viral PDB entries. The large increase shown from 1984 was due to the release of the tomato bushy stunt virus (PDB id: 2tbv) [13]. (A) Total number of entries, (B) details of viruses reported more than 50 times.

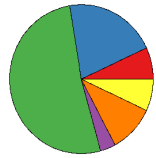
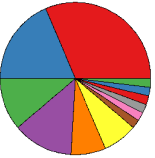
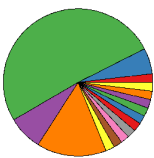
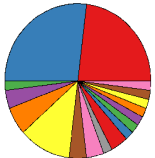
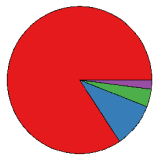
For each top virus a sequence cluster analysis (**Table 1**) was performed using a sequence identity of 50% as cut-off. More clusters than expected were obtained, as their number is bigger than the number of proteins of each viral proteome.

	<b>HIV-1</b>	<b>Enterobacteria phage</b>	<b>Influenza A virus</b>	<b>HCV</b>	<b>Human herpesvirus</b>
PDB entries	1617	1318	662	377	276
Clusters					
	108	242	47	32	78

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	<b>SARS-CoV</b>	<b>Dengue virus</b>	<b>Norwalk virus</b>	<b>Vaccinia virus</b>	<b>Enterovirus C</b>
PDB entries	170	127	110	110	84
Clusters					
	37	19	7	40	10

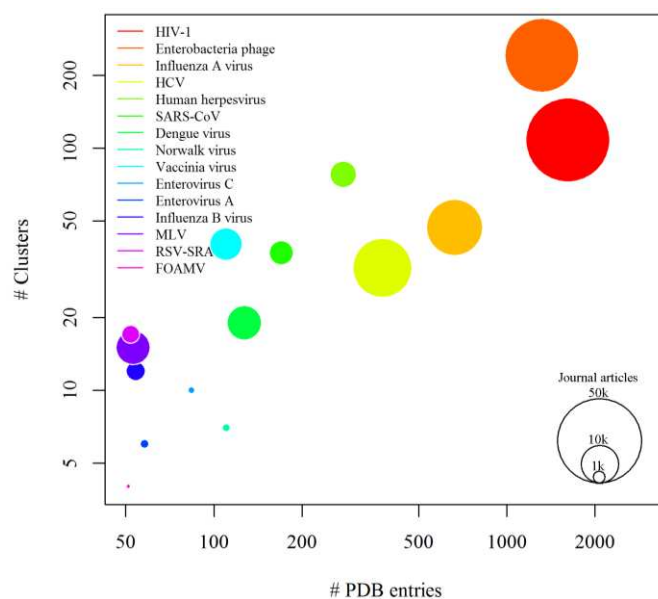
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	<b>Enterovirus A</b>	<b>Influenza B virus</b>	<b>MLV</b>	<b>RSV-SRA</b>	<b>Simian foamy virus</b>
PDB entries	58	54	53	52	51
Clusters					
	6	12	15	17	4

**Table 1.** Diversity of PDB entries. All viral PDB entries were analysed using a sequence identity of 50% as cut-off to identify clusters.

Biomedical research on viruses does not involve only structural biology, as highlighted in **Figure 3**, where the general interest of scientists is compared with the entries of viruses in the PDB. A search in the PubMed database using each virus name revealed the number of original research papers published

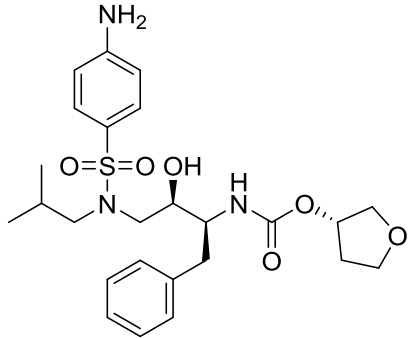
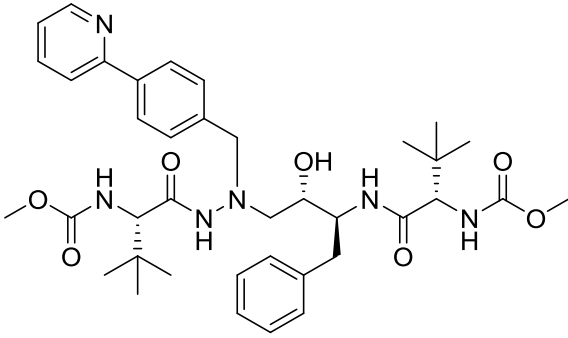
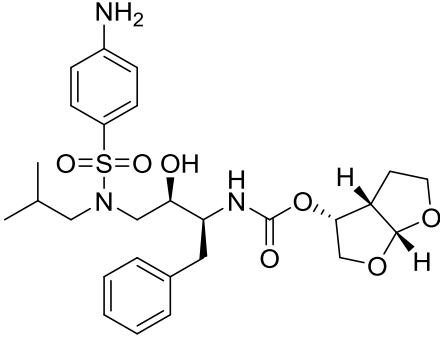
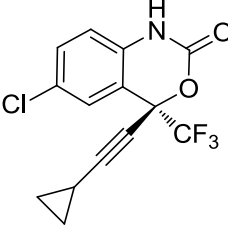
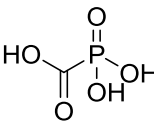
per virus. Surprisingly, the growth rate of publications in PubMed is not always correlated with the number of PDB entries.

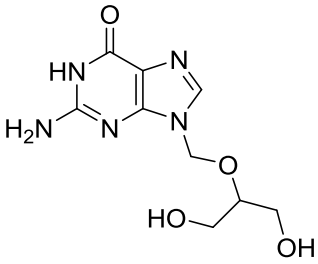
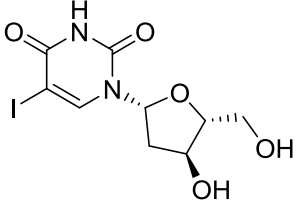
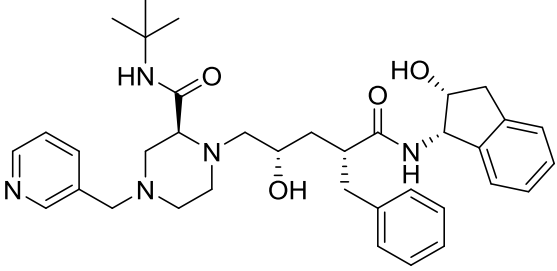
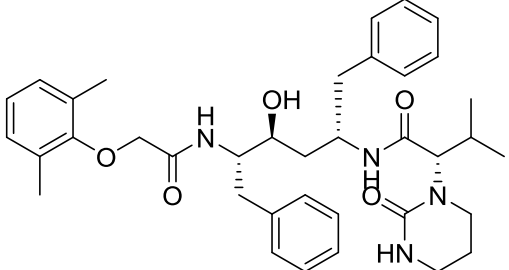
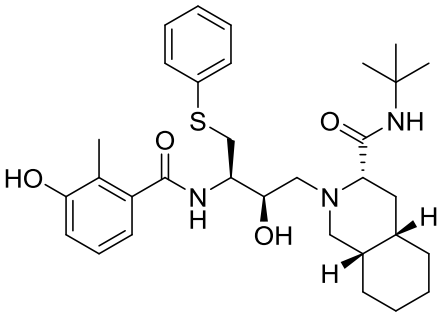
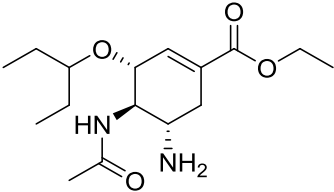


**Figure 3.** Analysis of the top viruses in the PDB versus the corresponding publications in PubMed.

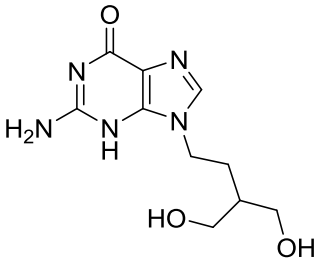
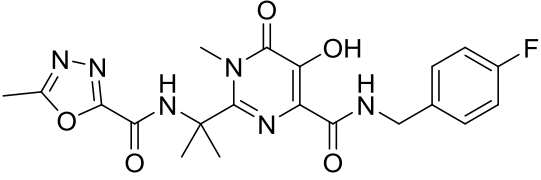
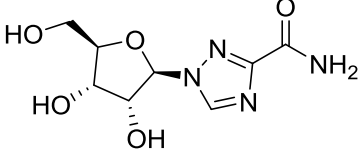
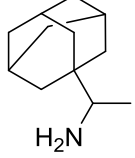
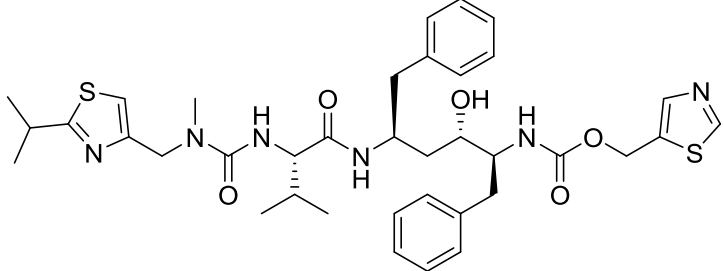
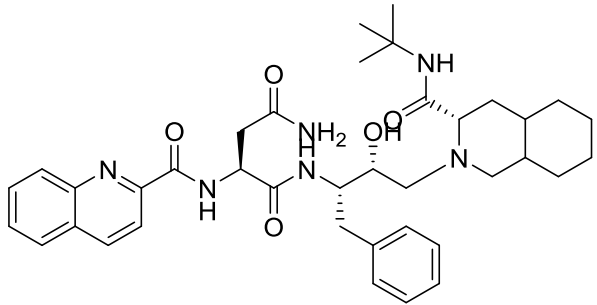
The number of ligand/viral PDB entries continues to increase; there are now more than 5,200 complexes with ligands, including different marketed drugs (**Table 2**).

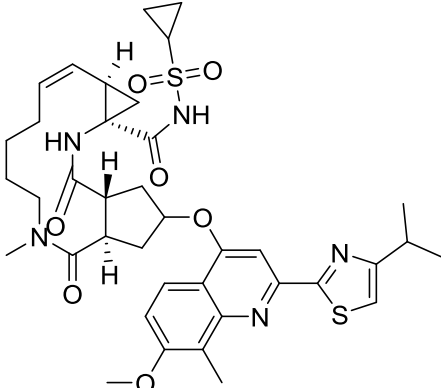
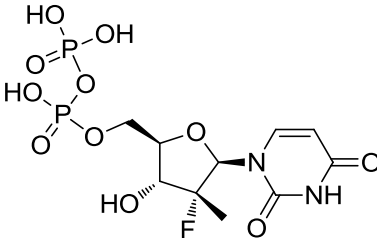
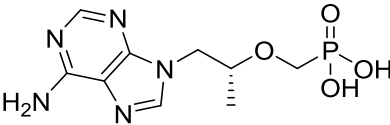
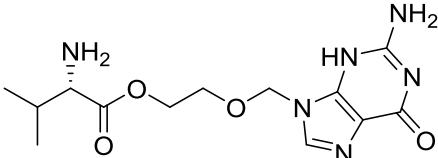
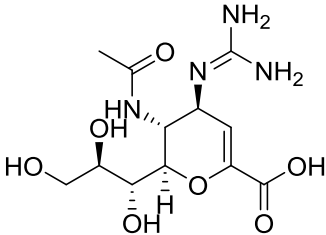
Name	Structure	PDB entry	Number of entries
Aciclovir	<chem>Nc1nc2nc(nc2c(=O)n1)COCCO</chem>	AC2	4
Amantadine	<chem>Nc1c2c(c1)C3CC4C(C2)CC(C3)C4</chem>	308	4

Name	Structure	PDB entry	Number of entries
Amprenavir		478	18
Atazanavir		DR7	10
Darunavir		017	49
Efavirenz (Sustiva)		EFZ	6
Foscarnet		PPF	7

Name	Structure	PDB entry	Number of entries
Ganciclovir		GA2	2
Idoxuridine		ID2	1
Indinavir		MK1	15
Lopinavir		AB1	10
Nelfinavir		1UN	10
Oseltamivir		G39	28



Name	Structure	PDB entry	Number of entries
Penciclovir		PE2	2
Raltegravir		RLT	4
Ribavirin		RBV	2
Rimantadine		RIM	2
Ritonavir		RIT	13
Saquinavir		ROC	25

Name	Structure	PDB entry	Number of entries
Simeprevir		30B	1
Sofosbuvir (diphosphate metabolite)		6GS	1
Tenofovir		TFO	1
Valaciclovir		TXC	1
Zanamivir		ZMR	20

**Table 2.** List of approved antiviral drugs currently available in the PDB in complex with their target.

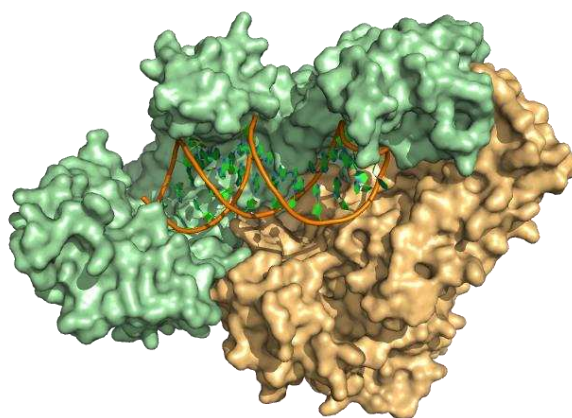
### 3. Recent applications of structural biology in antiviral research

One of the most striking examples of successful application of structural biology for antiviral drug discovery is represented by HIV-1 protease and reverse-transcriptase inhibitors. HIV-1 was recognised as the responsible for the acquired immune deficiency syndrome (AIDS) in the early 80s [18], and the discovery of the first selective HIV-1 protease inhibitors is still one of the most popular examples of the use of X-ray crystallography in the development of a drug in clinical use. The HIV protease was validated as a potential drug target in 1985 [19,20], the first X-ray crystal structures of the enzyme began appearing in 1989 [21-24] and the first HIV protease inhibitor Saquinavir was licensed only six years later, followed by the approval of Ritonavir four months later [25]. Three fundamental steps led to Saquinavir discovery,

the first one being the classification of HIV protease as a member of the aspartate protease family, which comprises also pepsin and renin [26]. Homology with renin, already a target in the design of anti-hypertensive agents, suggested a potential approach for the development of selective inhibitors of this enzyme [26], and this research interest was reinforced by the resolution of the first crystal structures for HIV [21,24], along with the protease structure of the related Rous sarcoma virus [22]. Finally, useful information on protease inhibition by transition-state analogue inhibitors [27-29] guided a series of investigations on the minimum size required for a small molecule to inhibit this enzyme. Extensive structure-activity relationship studies and X-ray experiments directed to address this aspect resulted in the discovery of Saquinavir.

A key step in HIV-1 life cycle is reverse transcription (RT), therefore the RT/DNA polymerisation has been immediately considered as a prime drug target, with the first approved anti-AIDS drug being the nucleoside analogue AZT (zidovudine, ZDV) in 1987 [30].

The HIV reverse transcriptase (RT) is a heterodimer consisting of two polypeptide chains, p66 and p51 (**Figure 4**). The p66 chain contains an N-terminal polymerase domain and a C-terminal RNase H domain [31,32]. The subdomains in p66 are flexible and can rearrange to different conformational states, required to carry out the enzyme essential functions for the viral replication. Sequencing of the complete RT from clinical isolates have shown that mutations in the remote connection subdomain and the RNase H domain enhance resistance to both nucleoside (NRTIs) and non-nucleoside (NNRTIs) inhibitors [33,34], following indirect mechanisms that are not well understood.



**Figure 4.** HIV-1 RT structure (PDB ID: 5D3G), p66 and p51 subunits are depicted in light green and light orange, respectively.

Since the publication of the first crystal structure of the HIV reverse-transcriptase in complex with the non-nucleoside inhibitor Nevirapine in 1994 [35], the resolution of the structures of several conformations of the complex with different inhibitors has provided an extremely powerful tool for gaining essential insights into the mechanism of action of this enzyme, for understanding the importance of its flexibility and its different conformational states, for rationalising the occurrence of resistance, for elucidating the binding of nucleoside and non-nucleoside inhibitors and for the design and optimisation of new chemical agents targeting this protein.

Among the several examples available on how structural biology has been essential for the design and optimisation of new non-nucleoside inhibitors of the HIV-1 RT, the resolution of a crystal structure of

the enzyme in complex with the RNase inhibitor dihydroxy benzoyl naphthyl hydrazone in 2006 (DHBNH, **Figure 5**) has led to the discovery of a novel site of the protein, near both the polymerase active site and the NNRTI binding pocket [36]. Structure-based modifications on the DHBNH scaffold resulted in the identification of dual inhibitors of both the polymerase and the RNH activities of the HIV-1 RT (exemplified by compound **1** in **Figure 5**).

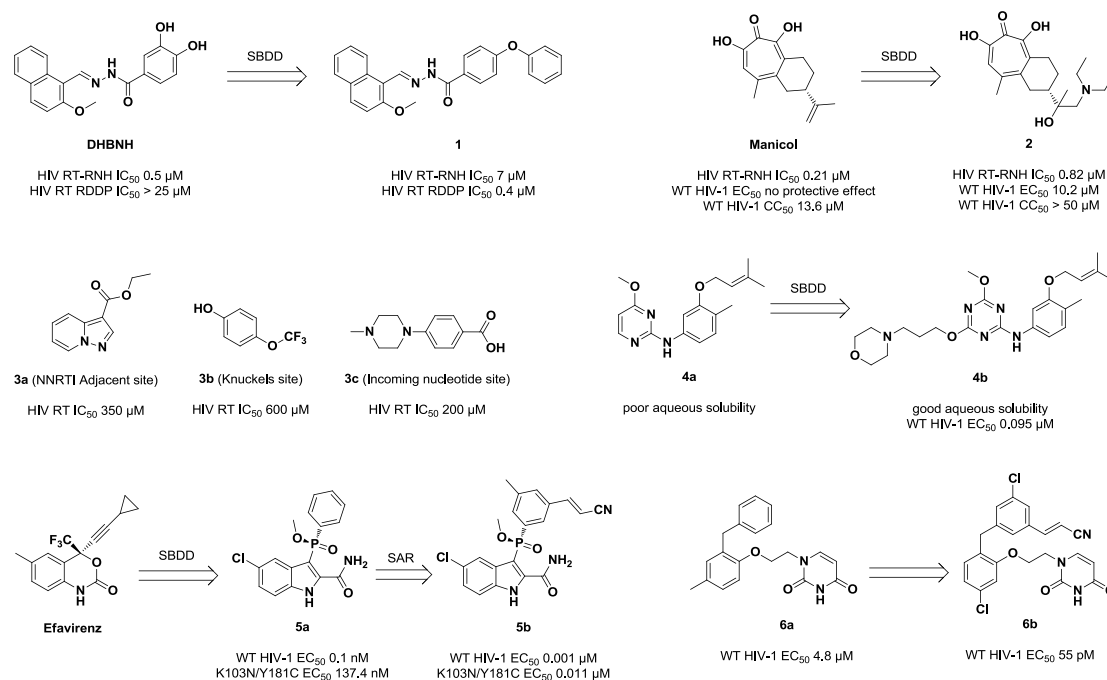
More recently, following the resolution of a crystal structure of HIV-1 RT containing the NNRTI TMC278 [37] in the DNA polymerase domain and  $\alpha$ -hydroxytropolone manicol in the RNase H active site, the structure of manicol was rationally modified to obtain a new series of  $\alpha$ -hydroxytropolones, which show antiviral activities at non-cytotoxic concentrations and occupy an additional site surrounding the DNA polymerase catalytic centre (compound **2** in **Figure 5**) [38].

In 2013, with the application of an X-ray crystallographic fragment screening methodology to evaluate the intrinsic flexibility of the RT for the discovery of new allosteric sites, seven new sites were identified within this protein [39]. Three of these sites (named the Knuckles, the NNRTI Adjacent and the Incoming Nucleotide binding sites) were proven inhibitory in an enzymatic assay, while the co-crystallised fragments (**3a-c** in **Figure 5**) were found to be novel scaffolds in comparison with previously reported RT inhibitors, thus providing the basis for the development of novel leads [39].

After the resolution of a crystal structure of HIV-1 RT in complex with potent pyrimidine-based NNRTI **4a**, structure-based modifications on its chemical scaffold directed to the occupation of the entrance channel to the NNRTI binding site resulted in the identification of much more soluble analogues such as **4b** (**Figure 5**), with which the solubility issues associated with **4a** were significantly improved [40].

In 2011, the study of several reported structures of the enzyme in complex with Efavirenz and other non-nucleoside inhibitors, and the inspection of the ligand geometries required for the interaction with the enzyme revealed in these structures, guided the design of a new series of aryl-phospho-indoles as potent inhibitors of the enzyme (exemplified by compound **5a** in **Figure 5**) [41]. Subsequent rational optimisation of the original lead **5a** resulted in the identification of **5b**, a nanomolar inhibitor of the Y181C/K103N double mutant (both mutations are clinically relevant) that reached phase IIb clinical trials [42].

In the same year, multiple RT crystal structures have been used for the *in silico* screening of a library of more than two million compounds using molecular docking methods, taking into account different protein conformations in order to overcome resistance [43]. One hit was found with 4.8  $\mu$ M potency against WT HIV-1 (compound **6a** in **Figure 5**). Computational analyses and rational modifications on the structure of **6a** led to the discovery of catechol diether **6b**, a 55-pM anti-HIV agent that retains nanomolar activity against the Y181C mutant [43].



**Figure 5:** Chemical structures of HIV-1 RT inhibitors discovered or optimised using structure-based methods.

Structural biology has often been extremely helpful for the identification or optimisation of novel antiviral agents also in the case of HCV, in particular for the discovery of novel inhibitors of the NS3-4a protein and the NS5b polymerase.

In 2012, a fragment-based screening of 176 fragments against the full length HCV NS3-NS4a genotype 1b enzyme led to the discovery of a new allosteric pocket at the interface between the protease and helicase domains [44]. Structure-based optimisation of a first hit found to bind this new pocket (compound **7a** in **Figure 6**) guided the identification of potent inhibitor **7b** (**Figure 6**), which also inhibits the viral replication with an  $\text{EC}_{50}$  value in the nanomolar range [44].

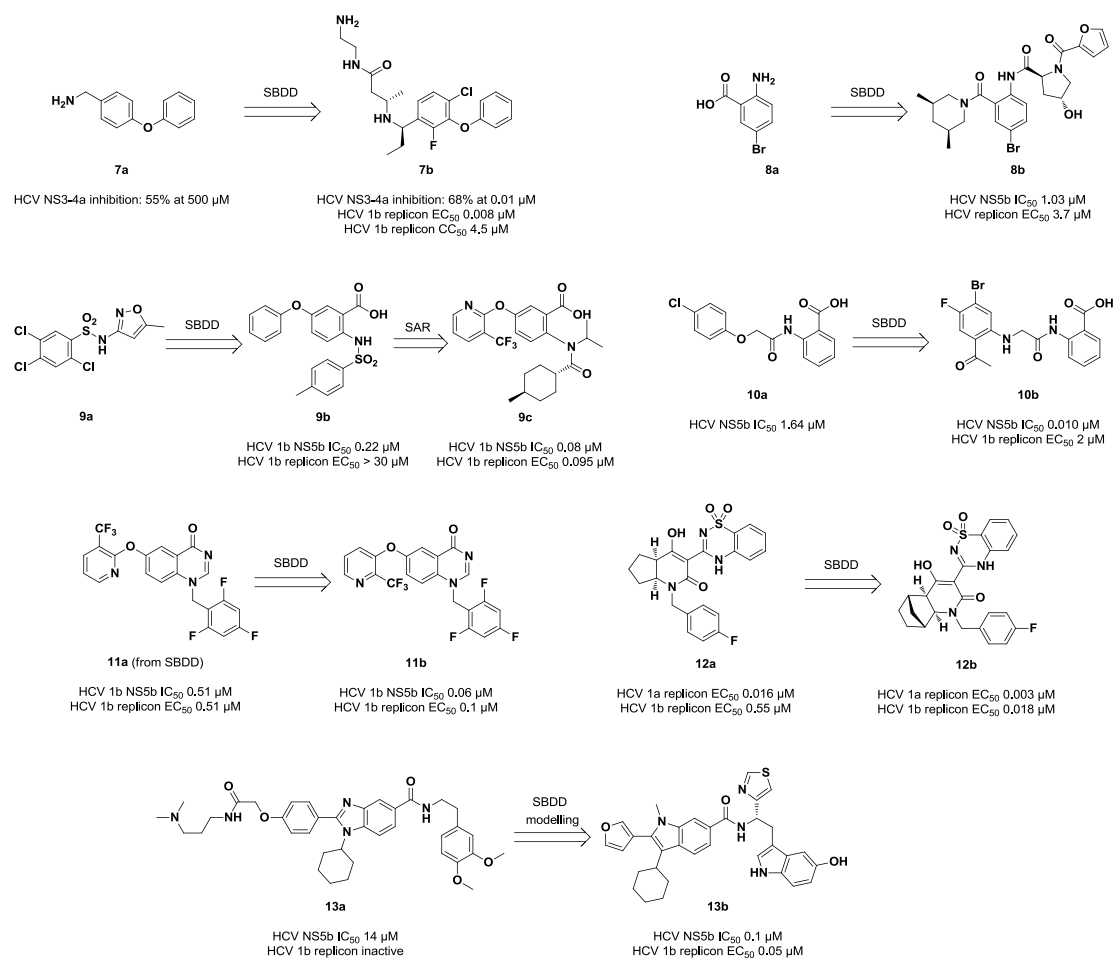
Crystallographic fragment-based screening methodologies have proven successful also in the identification of non-nucleoside inhibitors of the HCV NS5b polymerase. In 2008, a small bromo-aryl fragment (**8a**) was found to bind the thumb domain of the protein with an initial binding affinity in the millimolar range [45]. A series of structure-based optimization cycles on the fragment scaffold led to the identification of a family of structures with high affinity for the enzyme and low micromolar activities in the HCV replicon assay (compound **8b** in **Figure 6**).

More recently, the application of a similar fragment-based approach guided the identification of sulfonamide fragment **9a** (**Figure 6**), which binds the polymerase allosteric thumb pocket 2 [46]. The scaffold of this small fragment was the starting point for different structure-based modifications, which resulted in the identification of phenoxyantranilic acid sulfonamide derivative **9b** as a 650-fold more potent inhibitor of the HCV NS5b polymerase [46]. Further structure-based optimisation attempts on the scaffold of **9b** led to the discovery of derivative **9c**, which is slightly more potent in inhibiting the enzyme and shows a much more potent inhibition of the viral replication in cell culture, with an  $\text{EC}_{50}$  < 100 nM [47].

Along with fragment-based screening methods, structure-based design and optimisation techniques have played an important role in the discovery of novel non-nucleoside inhibitors of the HCV NS5b polymerase. One of the first of these studies was reported in 2007, when the structure of the non-nucleoside inhibitor **10a**, a hit identified by a biochemical HTS assay, was resolved in complex with the enzyme, revealing that it binds to an allosteric region between the thumb and palm domains [48]. Starting from the examination of this crystal structure, a series of rational modifications on the hit scaffold, directed to a better occupation of the allosteric pocket, resulted in the discovery of potent inhibitor **10b** (**Figure 6**), with  $IC_{50} < 17$  nM and activities against the viral replication in cellular systems in the low micromolar range [48].

Other recent examples of structure-based lead optimisation include the identification of a novel quinazolinone chemotype as thumb pocket 2 allosteric inhibitor (compound **11a** in **Figure 6**), which has been rationally designed following inspection of the enzyme crystal structures in complex with previously reported allosteric inhibitors [49]. After the crystal structure of the newly designed quinazolinone **11a** in complex with the enzyme was resolved, further structure-based optimisation attempts aiming to improve the key interactions within the protein binding channel were carried out, resulting in the identification of derivative **11b**, which shows improved potency in both the biochemical and the cellular antiviral assays (**Figure 6**) [49]. Finally, starting from the crystal structure of non-nucleoside inhibitor **12a** in complex with the polymerase, synthetic efforts directed towards the optimisation of the interactions with different sub-pockets of the enzyme resulted in the identification of several new analogues with potent antiviral activities *in vitro* against both genotype 1a and 1b, high metabolic stability and good oral bioavailability (represented by **12b** in **Figure 6**) [50].

Another interesting case of application of structural biology to the identification of HCV NS5B non-nucleoside inhibitors has been reported in 2010, when the structure of compound **13a**, an attractive hit deriving from a biochemical HTS assay of a large compound collection, was sequentially optimised using a combination of X-ray crystallography, NMR analyses and molecular modelling studies, along with binding-site resistance mutant experiments and photoaffinity labelling studies [51]. This approach resulted in the identification of different series of new analogues with significantly improved potency in both the enzymatic assay and a cellular antiviral assay (exemplified by compound **13b** **Figure 6**).

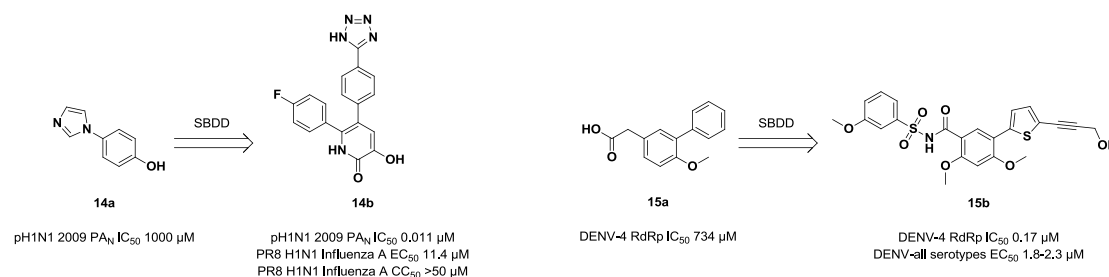


**Figure 6:** Chemical structures of non-nucleoside inhibitors of the HCV NS5B polymerase discovered or optimised with structure-based methods.

As mentioned above, there are many cases in which structural biology has been key to the identification of novel and improved antivirals, involving many of the viruses for which structural information has become available in the last decades, and including examples of antiviral drugs on the market, such as the neuraminidase inhibitor Zanamivir for influenza A and B [8]. Another example of successful application of a structure-based approach has been recently reported for the identification of a new class of influenza endonuclease inhibitors (**Figure 7**) [52]. An engineered high-resolution crystal form of pandemic 2009 influenza polymerase acidic protein N-terminal endonuclease domain was used for the crystallographic fragment screening of 775 fragments, leading to the identification of hit fragment **14a**, which showed a binding affinity to the enzyme in the range of 1000  $\mu$ M and also revealed the presence of a third metal ion in the active site cleft, previously unknown. Different cycles of rational modifications on the hit scaffold were performed in order to maximise the interactions with the active site of the enzyme. This structure-based optimisation approach resulted in the identification of compound **14b**, which shows an antiviral  $EC_{50}$  of 11  $\mu$ M (**Figure 7**).

Structural biology has been fundamental also to understand the mechanism of action and the importance of conformational flexibility of the Dengue Virus polymerase [53-55], and structure-based methods have been extremely useful for the discovery of non-nucleoside inhibitors of this enzyme. An X-ray-based screen of the Novartis fragment collection against DENV-3 polymerase resulted in the identification of

biphenyl acetic acid **15a** (**Figure 7**) bound to a novel pocket in the palm subdomain of the protein [56]. Growing and optimisation of this first hit through crystallography and computer-aided structure-based design led to the development of **15b**, an antiviral agent active against all four Dengue serotypes with EC<sub>50</sub> values in the low micromolar range.

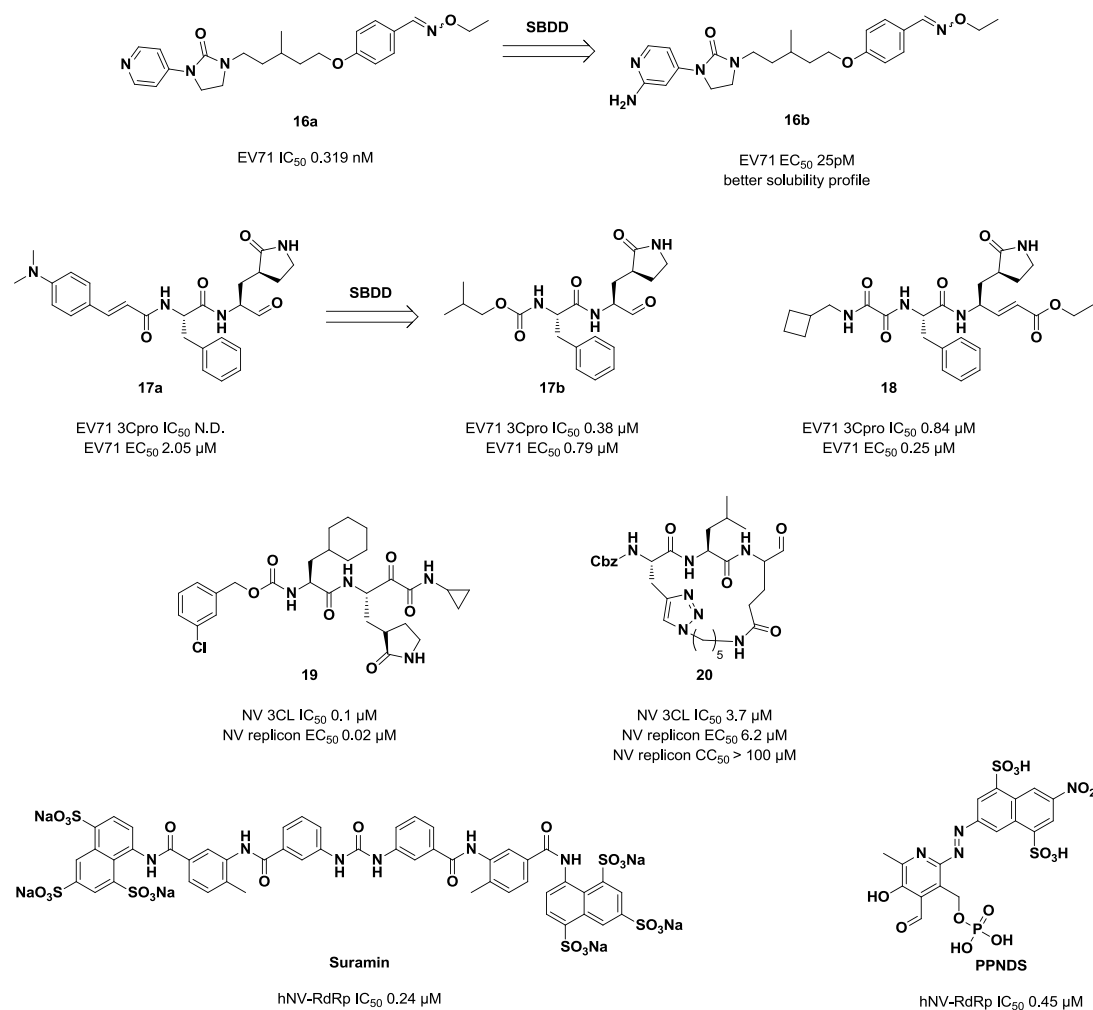


**Figure 7:** Chemical structures of influenza endonuclease inhibitors **14a-b** and Dengue polymerase inhibitors **15a-b**.

Among other examples, structure-based methods have been particularly useful for the discovery of novel chemical agents against enterovirus 71 and norovirus. In 2014, the analysis of the crystal structures of the EV71 complete viral particle in complex with uncoating inhibitors (exemplified by derivative **16a** in **Figure 8**), in combination with *in silico* docking-based methods, guided the design of an improved and potent inhibitor of the virus-induced cytopathic effect in cells (compound **16b**), which inhibits the full range of EV71 subtypes [57]. More recently, the analysis of the crystal structure of the EV71 3C proteinase in complex with moderate inhibitor **17a**, and the subsequent structure-based modification of its scaffold, guided the design of **17b**, an improved inhibitor in both the enzymatic and a virus cell-based assay [58]. Finally, a structure-based fragment-wise design of new inhibitors of the same enzyme led to the discovery of the new chemical scaffold **18** as a potent inhibitor of both the enzyme activity and the virus-induced cytopathic effect in cells [59].

In the case of norovirus, an iterative process of structure-guided design and optimisation on dipeptidyl inhibitors of the viral 3C-like protease has guided the discovery of potent derivative **19**, which displays *in vivo* efficacy in a murine model of norovirus infection [60]. For the study of the same viral target, different crystal structures in complex with peptidyl inhibitors have recently been used to design novel triazole-based macrocyclic inhibitors (represented by compound **20** in **Figure 8**), which also inhibit the viral replication in cells with EC<sub>50</sub> values in the low micromolar range [61]. Finally, structural biology has been essential also for the identification of novel inhibitors of norovirus RNA-dependent RNA-polymerase. A docking-based *in silico* search method on the polymerase structure using commercially available compounds led to the discovery of suramin and its analogues [62] and of PPNS [63, 64] as novel inhibitors of the norovirus RdRp activity. The resolution of the crystal structure of PPNS in complex with the enzyme has also indicated the possibility to target a new binding sub-pocket in the thumb domain of the enzyme for the design of new norovirus inhibitors [64].





**Figure 8:** Chemical structures of enterovirus 71 and norovirus inhibitors discovered with the aid of structural methods.

## 4. Conclusions

Following the increasing number of viral target structures deposited in the PDB, structural biology has become a fundamental tool in antiviral research, not only providing essential insights into the mechanisms of actions of viral enzymes and their interactions with substrates and antiviral agents, but representing in several cases the very basis for the rational discovery and optimisation of new antivirals, as exemplified by the striking case of drugs in the market such as Saquinavir and Zanamivir. Used in combination with computational techniques, structure-based methods are often among the earliest and most important steps of drug-discovery campaigns of most pharmaceutical companies, and their fundamental application for the discovery of new antivirals can only be expected to increase over the next years, supported by the continuous evolution of associated technologies.

## 5. Highlights

Resolution of three-dimensional viral structures in the PDB statistically analysed.

Evolution of structure-based methods in antiviral drug-discovery overviewed.

Different examples of the role of structural biology in the discovery and optimisation of new antivirals examined.

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## 8. References

1. Blundell TL, Jhoti H, Abell C: **High-throughput crystallography for lead discovery in drug design.** *Nature reviews Drug discovery* (2002) **1**(1):45-54.
2. Howard JA: **Dorothy hodgkin and her contributions to biochemistry.** *Nature reviews Molecular cell biology* (2003) **4**(11):891-896.
3. Tang L, Johnson JE: **Structural biology of viruses by the combination of electron cryomicroscopy and x-ray crystallography.** *Biochemistry* (2002) **41**(39):11517-11524.
4. Talele TT, Khedkar SA, Rigby AC: **Successful applications of computer aided drug discovery: Moving drugs from concept to the clinic.** *Current topics in medicinal chemistry* (2010) **10**(1):127-141.
5. Congreve M, Murray CW, Blundell TL: **Structural biology and drug discovery.** *Drug discovery today* (2005) **10**(13):895-907.
6. Pearl LH, Taylor WR: **A structural model for the retroviral proteases.** *Nature* (1987) **329**(6137):351-354.
7. Blundell T, Carney D, Gardner S, Hayes F, Howlin B, Hubbard T, Overington J, Singh DA, Sibanda BL, Sutcliffe M: **18th sir hans krebs lecture. Knowledge-based protein modelling and design.** *European journal of biochemistry / FEBS* (1988) **172**(3):513-520.
8. Varghese JN: **Development of neuraminidase inhibitors as anti-influenza virus drugs.** *Drug Development Research* (1999) **46**(3-4):176-196.
9. Russell RB, Eggleston DS: **New roles for structure in biology and drug discovery.** *Nature structural biology* (2000) **7 Suppl**(928-930).

10. Nienaber VL, Richardson PL, Klighofer V, Bouska JJ, Giranda VL, Greer J: **Discovering novel ligands for macromolecules using x-ray crystallographic screening.** *Nat Biotech* (2000) **18**(10):1105-1108.
11. Hardy LW, Malikayil A: **The impact of structure-guided drug design on clinical agents.** *Curr Drug Discov* (2003) 15-20.
12. Berman HM, Kleywegt GJ, Nakamura H, Markley JL: **The protein data bank at 40: Reflecting on the past to prepare for the future.** *Structure* (2012) **20**(3):391-396.
13. Harrison SC, Olson AJ, Schutt CE, Winkler FK, Bricogne G: **Tomato bushy stunt virus at 2.9 a resolution.** *Nature* (1978) **276**(5686):368-373.
14. Lane SW, Dennis CA, Lane CL, Trinh CH, Rizkallah PJ, Stockley PG, Phillips SE: **Construction and crystal structure of recombinant stnv capsids.** *Journal of molecular biology* (2011) **413**(1):41-50.
15. Sanchez-Weatherby J, Bowler MW, Huet J, Gobbo A, Felisaz F, Lavault B, Moya R, Kadlec J, Ravelli RB, Cipriani F: **Improving diffraction by humidity control: A novel device compatible with x-ray beamlines.** *Acta crystallographica Section D, Biological crystallography* (2009) **65**(Pt 12):1237-1246.
16. Pereira JH, Ralston CY, Douglas NR, Meyer D, Knee KM, Goulet DR, King JA, Frydman J, Adams PD: **Crystal structures of a group ii chaperonin reveal the open and closed states associated with the protein folding cycle.** *The Journal of biological chemistry* (2010) **285**(36):27958-27966.
17. Kern J, Alonso-Mori R, Hellmich J, Tran R, Hattne J, Laksmono H, Glockner C, Echols N, Sierra RG, Sellberg J, Lassalle-Kaiser B *et al*: **Room temperature femtosecond x-ray diffraction of photosystem ii microcrystals.** *Proceedings of the National Academy of Sciences of the United States of America* (2012) **109**(25):9721-9726.
18. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W *et al*: **Isolation of a t-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (aids).** *Science* (1983) **220**(4599):868-871.

19. Katoh I, Yoshinaka Y, Rein A, Shibuya M, Odaka T, Oroszlan S: **Murine leukemia virus maturation: Protease region required for conversion from "immature" to "mature" core form and for virus infectivity.** *Virology* (1985) **145**(2):280-292.
20. Crawford S, Goff SP: **A deletion mutation in the 5' part of the pol gene of moloney murine leukemia virus blocks proteolytic processing of the gag and pol polypeptides.** *Journal of virology* (1985) **53**(3):899-907.
21. Lapatto R, Blundell T, Hemmings A, Overington J, Wilderspin A, Wood S, Merson JR, Whittle PJ, Danley DE, Geoghegan KF, et al.: **X-ray analysis of hiv-1 proteinase at 2.7 a resolution confirms structural homology among retroviral enzymes.** *Nature* (1989) **342**(6247):299-302.
22. Weber IT, Miller M, Jaskolski M, Leis J, Skalka AM, Wlodawer A: **Molecular modeling of the hiv-1 protease and its substrate binding site.** *Science* (1989) **243**(4893):928-931.
23. Wlodawer A, Miller M, Jaskolski M, Sathyanarayana BK, Baldwin E, Weber IT, Selk LM, Clawson L, Schneider J, Kent SB: **Conserved folding in retroviral proteases: Crystal structure of a synthetic hiv-1 protease.** *Science* (1989) **245**(4918):616-621.
24. Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Krohn A, et al.: **Rational design of peptide-based hiv proteinase inhibitors.** *Science* (1990) **248**(4953):358-361.
25. Wlodawer A: **Structure-based design of aids drugs and the development of resistance.** *Vox sanguinis* (2002) **83 Suppl 1**: 23-26.
26. Katoh I, Yasunaga T, Ikawa Y, Yoshinaka Y: **Inhibition of retroviral protease activity by an aspartyl proteinase inhibitor.** *Nature* (1987) **329**(6140):654-656.
27. Szelke M, Leckie B, Hallett A, Jones DM, Sueiras J, Atrash B, Lever AF: **Potent new inhibitors of human renin.** *Nature* (1982) **299**(5883):555-557.
28. Boger J, Lohr NS, Ulm EH, Poe M, Blaine EH, Fanelli GM, Lin TY, Payne LS, Schorn TW, LaMont BI, Vassil TC *et al*: **Novel renin inhibitors containing the amino acid statine.** *Nature* (1983) **303**(5912):81-84.
29. Allen MC, Fuhrer W, Tuck B, Wade R, Wood JM: **Renin inhibitors. Synthesis of transition-state analogue inhibitors containing phosphorus acid derivatives at the scissile bond.** *Journal of medicinal chemistry* (1989) **32**(7):1652-1661.

30. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT, et al.: **The efficacy of azidothymidine (azt) in the treatment of patients with aids and aids-related complex. A double-blind, placebo-controlled trial.** *The New England journal of medicine* (1987) **317**(4):185-191.
31. Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA: **Crystal structure at 3.5 a resolution of hiv-1 reverse transcriptase complexed with an inhibitor.** *Science* (1992) **256**(5065):1783-1790.
32. Jacobo-Molina A, Ding J, Nanni RG, Clark AD, Jr., Lu X, Tantillo C, Williams RL, Kamer G, Ferris AL, Clark P, et al.: **Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 a resolution shows bent DNA.** *Proceedings of the National Academy of Sciences of the United States of America* (1993) **90**(13):6320-6324.
33. Nikolenko GN, Palmer S, Maldarelli F, Mellors JW, Coffin JM, Pathak VK: **Mechanism for nucleoside analog-mediated abrogation of hiv-1 replication: Balance between rnase h activity and nucleotide excision.** *Proceedings of the National Academy of Sciences of the United States of America* (2005) **102**(6):2093-2098.
34. Yap SH, Sheen CW, Fahey J, Zanin M, Tyssen D, Lima VD, Wynhoven B, Kuiper M, Sluis-Cremer N, Harrigan PR, Tachedjian G: **N348i in the connection domain of hiv-1 reverse transcriptase confers zidovudine and nevirapine resistance.** *PLoS medicine* (2007) **4**(12):e335.
35. Smerdon SJ, Jäger J, Wang J, Kohlstaedt LA, Chirino AJ, Friedman JM, Rice PA, Steitz Ta. **Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of human immunodeficiency virus type 1.** *Proceedings of the National Academy of Sciences of the United States of America* (1994) **91**(9):3911-3915.
36. Himmel DM, Sarafianos SG, Dharmasena S, Hossain MM, McCoy-Simandle K, Ilina T, Clark AD Jr., Knight JL, Julias JG, Clark PK, Krogh-Jespersen K, Levy RM, Hughes SH, Parniak MA, Arnold E. **HIV-1 Reverse transcriptase structure with RNase H inhibitor dihydroxy benzoyl naphthyl hydrazone bound at a novel site.** *ACS Chemical Biology* (2006), **1**(11):702-712.
37. Boone LR. **Next-generation HIV-1 non-nucleoside reverse transcriptase inhibitors.** *Current Opinion in Investigational Drugs.* (2006) **7**(2):128–135.

38. Chung S, DanHimmel DM, Jiang J-K, Wojtak K, Bauman JD, Rausch JW, Wilson JA, Beutler JA, Thomas CJ, Arnold E, Le Grice SFJ. **Synthesis, activity and structural analysis of novel  $\alpha$ -hydroxytropolone inhibitors of human immunodeficiency virus reverse transcriptase-associated ribonuclease H.** *Journal of Medicinal Chemistry* (2011) **54**(13): 4462-4473.
39. Bauman JD, Patel D, Dharia C, Fromer MW, Ahmed S, Frenkel Y, Vijayan RSK, Eck JT, Ho WC, Das K, Shatkin AJ, Arnold E. **Detecting allosteric sites of HIV-1 reverse transcriptase by X-ray crystallographic fragment screening.** *Journal of Medicinal Chemistry* (2013) **56**(7): 2738-2746.
40. Bollini M, Frey KM, Cisneros JA, Spasov KA, Das K, Bauman JD, Arnold E, Anderson KS, Jorgensen WL. **Extension into the entrance channel of HIV-1 reverse transcriptase—Crystallography and enhanced solubility.** *Bioorganic & Medicinal Chemistry Letters* (2013) **23**(18): 5209–5212.
41. Alexandre FE, Amador A, Bot S, Caillet C, Convard T, Jakubik J, Musiu C, Poddesu B, Vargiu L, Liuzzi M, Roland A, Seifer M, Standring D, Storer R, Dousson CB. **Synthesis and biological evaluation of aryl-phospho-indole as novel HIV-1 non-nucleoside reverse transcriptase inhibitors.** *Journal of Medicinal Chemistry* (2011) **54**(1): 392-395.
- 42.\*\* Dousson C, Alexandre FR, Amador A, Bonaric S, Bot S, Caillet C, Convard T, da Costa D, Lioure MP, Roland A, Rosinovsky E, Maldonado S, Parsy C, Trochet C, Storer R, Stewart A, Wang J, Mayes BA, Musiu C, Poddesu B, Vargiu L, Liuzzi M, Moussa A, Jakubik J, Hubbard L, Seifer M, Standring D. **Discovery of the aryl-phospho-indole IDX899, a highly potent anti-HIV non-nucleoside reverse transcriptase inhibitor.** *Journal of Medicinal Chemistry* (2016) **59**(5): 1891-1898.  
  
This paper represents an excellent example of the use of structural biology in the discovery of new antivirals against HIV.
43. Bollini M, Domaoal DA, Thakur VV, Gallardo-Macias R, Spasov KA, Anderson KS, Jorgensen WL. **Computationally-guided optimization of a docking hit to yield catechol diethers as potent anti-HIV agents.** *Journal of Medicinal Chemistry* (2011) **54**(24): 8582-8591.
44. Saalau-Bethell SM, Woodhead AJ, Chessari G, Carr MG, Coyle J, Graham B, Hiscock SD, Murray CW, Pathuri P, Rich SJ, Richardson CJ, Williams PA, Jhoti H. **Discovery of an allosteric mechanism for the regulation of HCV NS3 protein function.** *Nature Chemical Biology* (2012) **8**(11): 920-925.
45. Antonysamy SS, Aubol B, Blaney J, Browner MF, Giannetti AM, Harris SF, Hébert N, Hendle J,

- Hopkins S, Jefferson E, Kissinger C, Leveque V, Marciano D, McGee E, Nájera I, Nolan B, Tomimoto M, Torresa E, Wright T. **Fragment-based discovery of hepatitis C virus NS5b RNA polymerase inhibitors.** *Bioorganic & Medicinal Chemistry Letters* (2008) **18**(9):2990–2995.
46. Stammers TA, Coulombe R, Rancourt J, Thavonekham B, Fazal G, Goulet S, Jakalian A, Wernic D, Tsantrizos Y, Poupart M-A, Börs M, McKercher G, Thauvette L, Kukolj G, Beaulieu PL. **Discovery of a novel series of non-nucleoside thumb pocket 2 HCV NS5B polymerase inhibitors.** *Bioorganic & Medicinal Chemistry Letters* (2013) **23**(9): 2585–2589.
  47. Stammers TA, Coulombe R, Duplessis M, Fazal G, Gagnon A, Garneau M, Goulet S, Jakalian A, LaPlante S, Rancourt J, Thavonekham B, Wernic D, Kukolj G, Beaulieu PL. **Anthranilic acid-based Thumb Pocket 2 HCV NS5B polymerase inhibitors with sub-micromolar potency in the cell-based replicon assay.** *Bioorganic & Medicinal Chemistry Letters* (2013) **23**(24): 6879–6885.
  48. Nittoli T, Curran K, Insaf S, DiGrandi M, Orlowski M, Chopra R, Agarwal A, Howe AYM, Prashad A, Brawner Floyd M, Johnson B, Sutherland A, Wheless K, Feld B, O'Connell J, Mansour TS, Bloom J. **Identification of anthranilic acid derivatives as a novel class of allosteric inhibitors of hepatitis C NS5B polymerase.** *Journal of Medicinal Chemistry* (2007) **50**(9): 2108-2116.
  49. Beaulieu PL, Coulombe R, Duan J, Fazal G, Godbout C, Hucke O, Jakalian A, Joly MA, Lepage O, Llinàs-Brunet M, Naud J, Poirier M, Rioux N, Thavonekham B, Kukolj G, Stammers TA. **Structure-based design of novel HCV NS5B thumb pocket 2 allosteric inhibitors with submicromolar gt1 replicon potency: Discovery of a quinazolinone chemotype.** *Bioorganic & Medicinal Chemistry Letters* (2013) **23**(14): 4132–4140.
  50. Ruebsam F, Murphy DE, Tran CV, Li L-S, Zhao J, Dragovich PS, McGuire HM, Xiang AX, Sun Z, Ayida BK, Blazel JK, Kim SH, Zhou Y, Han Q, Kissinger CR, Webber SE, Showalter RE, Shah AM, Tsan M, Patel RA, Thompson PA, LeBrun LA, Hou HJ, Kamran R, Sergeeva MV, Bartkowski DM, Nolan TG, Norris DA, Khandurina J, Brooks J, Okamoto E, Kirkovsky L. **Discovery of tricyclic 5,6-dihydro-1H-pyridin-2-ones as novel, potent, and orally bioavailable inhibitors of HCV NS5B polymerase.** *Bioorganic & Medicinal Chemistry Letters* (2009) **19**(22): 6404–6412.
  51. LaPlante SR, Gillard JR, Jakalian A, Aubry N, Coulombe R, Brochu C, Tsantrizos YS, Poirier M, Kukolj G, Beaulieu PL. **Importance of ligand bioactive conformation in the discovery of potent indole-diamide inhibitors of the hepatitis C virus NS5B.** *Journal of the American Chemical Society* (2010) **132**(43): 15204-15212.

52. Bauman JD, Patel D, Baker SF, Vijayan RSK, Xiang A, Parhi AK, Martínez-Sobrido L, LaVoie EJ, Das K, Arnold E. **Crystallographic fragment screening and structure-based optimization yields a new class of Influenza endonuclease inhibitors.** *ACS Chemical Biology* (2013) **8**(11): 2501-2508.
53. Malet H, Massé N, Selisko B, Romette J-L, Alvarez K, Guillemot JC, Tolou H, Yap TL, Vasudevan SG, Lescar J, Canard B. **The flavivirus polymerase as a target for drug discovery.** *Antiviral Research* (2008) **80**(1): 23–35.
54. Noble CG, Lim SP, Chen YL, Liew CW, Yap L, Lescar J, Shi P-Y. **Conformational flexibility of the Dengue Virus RNA-Dependent RNA Polymerase revealed by a complex with an inhibitor.** *Journal of Virology* (2013) **87**(9): 5291-5295.
- 55.\* Zhao Y, Soh S, Zheng J, Chan KWK, Phoo WW, Lee CC, Tay MYF, Swaminathan K, Cornvik TC, Lim SP, Shi P, Lescar J, Vasudevan SG, Luo D. **A crystal structure of the dengue virus NS5 protein reveals a novel inter-domain interface essential for protein flexibility and virus replication.** *PLOS Pathogens* (2015) **11**(3): e1004682.  
This paper represents a significant example of the use of structural biology for understanding the mechanism of action of viral enzymes and the importance of their conformational flexibility, and for discovering new inhibitors.
- 56.\*\* Yokokawa F, Nilar S, Noble CG, Lim SP, Rao R, Tania S, Wang G, Lee G, Hunziker J, Karuna R, Manjunatha U, Shi P-Y, Smith PW. **Discovery of potent non-nucleoside inhibitors of Dengue viral RNA dependent RNA polymerase from a fragment hit using structure-based drug design.** *Journal of Medicinal Chemistry* (2016) **59**(8): 3935-3952.  
This paper represents an excellent example of the importance of structural biology for the discovery of novel antivirals.
- 57.\* De Colibus L, Wang X, Spyroul JAB, Kelly J, Ren J, Grimes J, Puerstinger P, Stonehouse N, Walter TS, Hu Z, Wang J, Li X, Peng W, Rowlands D, Fry EE, Rao Z, Stuart DI. **More powerful virus inhibitors from structure-based analysis of HEV71 capsid-binding molecules.** *Nature Structural & Molecular Biology* (2014) **21**(3): 282-288.  
This paper represents a significant example of the importance of structural biology for the rational optimisation of antiviral compounds.
- 58.\* Zhanga L, Huang G, Caia Q, Zhaoa C, Tanga L, Rena H, Lib P, Lib N, Huangc J, Chend X, Guanf Y, Youa H, Chenb S, Lib J, Lin T. **Optimize the interactions at S4 with efficient inhibitors targeting 3C proteinase from enterovirus 71.** *Journal of Molecular Recognition* (2016) Ahead of print.



This paper represents a significant example of the importance of structural biology for the rational optimisation of antiviral compounds.

- 59.\* Wu C, Zhang L, Li P, Cai Q, Peng X, Yinc K, Chen X, Ren H, Zhong S, Wengd Y, Guan Y, Chen S, Wua J, Li J, Lin T. **Fragment-wise design of inhibitors to 3C proteinase from enterovirus 71.** *Biochimica et Biophysica Acta* (2016) **1860**(6): 1299-1307.

This paper represents a significant example of the importance of structural methods for identifying novel antivirals.

- 60.\*\* Galasiti Kankanamalage AC, Kim Y, Weerawarna PM, Uy RAZ, Damalanka VC, Rao Mandadapu S, Alliston KR, Mehzabeen N, Battaile KP, Lovell S, Chang OK, Groutas WC. **Structure-guided design and optimization of dipeptidyl inhibitors of Norovirus 3CL protease. Structure-activity relationships and biochemical, X-ray crystallographic, cell-based, and in vivo studies.** *Journal of Medicinal Chemistry* (2015), **58**(7): 3144-3155.

This paper represents an excellent example of the use of structural methods for designing novel antivirals.

- 61.\* Weerawarna PM, Kim Y, Galasiti Kankanamalage AC, Damalanka VC, Lushington GH, Alliston KR, Mehzabeen N, Battaile KP, Lovell S, Chang KO, Groutas WC. **Structure-based design and synthesis of triazole-based macrocyclic inhibitors of norovirus protease: Structural, biochemical, spectroscopic, and antiviral studies.** *European Journal of Medicinal Chemistry* (2016), **119**: 300-318.

This paper represents a significant example of the use of structure-based methods for the discovery of novel antivirals.

62. Mastrangelo E, Pezzullo M, Tarantino D, Petazzi R, Germani F, Kramer D, Robel I, Rohayem J, Bolognesi M, Milani M. **Structure-based inhibition of Norovirus RNA-dependent RNA-polymerase.** *Journal of Molecular Biology* (2012), **419**(3-4): 198-210.

63. Croci R, Tarantino D, Milani M, Pezzullo M, Rohayem J, Bolognesi M, Mastrangelo E. **PPNDS inhibits murine Norovirus RNA-dependent RNA-polymerase mimicking two RNA stacking bases.** *FEBS Letters* (2014), **588**(9): 1720-1725.

64. Tarantino D, Pezzullo M, Mastrangelo E, Croci R, Rohayem J, Robel I, Bolognesi M, Milani M. **Naphthalene-sulfonate inhibitors of human norovirus RNA-dependent RNA-polymerase.** *Antiviral Research* (2014), **102**: 23-28.

